Interaction between AIF and CHCHD4 regulates respiratory chain biogenesis

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Running title:
Regulation of the respiratory chain by AIF/CHCHD4

Highlights:

- AIF interacts with CHCHD4 a regulator of the intermembrane space import machinery
- AIF regulates specific respiratory chain complexes by acting upstream of CHCHD4
- AIF is indispensable for translation-coupled mitochondrial import of CHCHD4
- Restoring CHCHD4 reverses the metabolic and cell death phenotypes of *Aif*$^{/y}$ ES cells

Summary

Apoptosis-inducing factor (AIF) is a phylogenetically conserved mitochondrial flavoprotein that, beyond its apoptotic function, is required for the normal expression of major respiratory chain complexes. Here, we identified a novel AIF-interacting protein, CHCHD4, which is the central component of a redox-sensitive mitochondrial intermembrane space import machinery. Depletion, deletion or hypomorphic mutation of AIF caused a reduction of the level of CHCHD4 protein by diminishing its mitochondrial import. CHCHD4 depletion and deletion sufficed to induce a respiratory defect that mimicked that observed in AIF-deficient cells. CHCHD4 levels could be restored in AIF-deficient cells by enforcing its AIF-independent mitochondrial localization. This modified CHCHD4 protein reestablished normal respiratory function in AIF-deficient cells and enabled AIF-deficient embryoid bodies to undergo cavitation, a process of programmed cell death required for embryonic morphogenesis. These findings explain how AIF contributes to the biogenesis of respiratory chain complexes and establish an unexpected link between the vital function of AIF and the propensity of cells to undergo apoptosis.

Introduction

Apoptosis-inducing factor (AIF) was initially characterized as a membrane-bound redox-active flavoprotein that is confined to the mitochondrial intermembrane space of healthy cells, yet translocates to the cytosol and the nucleus upon the apoptosis-associated induction of mitochondrial outer membrane permeabilization (MOMP) (Susin et al., 1999) coupled to its proteolysis by calpains (Norberg et al., 2010). AIF has been involved in the regulation and execution of apoptosis throughout eukaryote phylogeny, in yeast, filamentous fungi, nematodes, flies and mammals (Hangen et al., 2010a; Joza et al., 2001). In the cytosol, AIF can signal for phosphatidylserine exposure on the plasma membrane (Susin et al., 1999). Moreover, in the nucleus, AIF can participate in chromatin condensation and caspase-independent large-scale DNA fragmentation (Susin et al., 1999), likely through direct electrostatic interaction with the DNA phosphodiester backbone (Mate et al., 2002; Ye et al., 2002), as well as with other proteins that possess latent DNase activities (Parrish and Xue, 2003). AIF-deficient cells are resistant against a restricted panel of cell death inducers. In particular, mouse embryonic stem (ES) cells lacking AIF due to homologous recombination ($Aif^{-/y}$) fail to undergo cavitation (Feraud et al., 2007; Joza et al., 2001; Qi et al., 2012), a process of cell loss within the early developing embryo that reflects the first wave of programmed cell death during ontogeny.

Beyond its role in apoptosis, AIF also participates in normal mitochondrial metabolism. Indeed, in all species investigated, the absence of AIF causes a respiratory chain defect (Brown et al., 2006; Hangen et al., 2010a; Pospisilik et al., 2007; Vahsen et al., 2004) that is coupled to the post-transcriptional downregulation of protein subunits belonging to respiratory chain complexes I, III and IV (Benit et al., 2008; Hangen et al., 2010a; Pospisilik et al., 2007; Vahsen et al., 2004). The degenerative phenotype of the mice affected by a hypomorphic AIF mutation ("Harlequin mice") (Klein et al., 2002) correlates strictly with a

complex I-related respiratory dysfunction (Benit et al., 2008). A similar phenotype can be reproduced by tissue-specific knockout of AIF in mice (Hangen et al., 2010a), and is observed in human infants with loss-of-function mutations of AIF (Berger et al., 2011; Ghezzi et al., 2010). Both Harlequin mice and AIF-deficient infants similarly develop severe neuromuscular mitochondriopathies leading to premature death (Berger et al., 2011; Ghezzi et al., 2010; Klein et al., 2002).

The mechanisms through which AIF affects the assembly or stability of respiratory chain complexes are unknown. Moreover, the relationship between AIF, respiratory function and cavitation is elusive. Here, we report the discovery of an AIF-interacting protein, CHCHD4 that provides a mechanistic link between AIF deficiency, mitochondrial dysfunction and failed cavitation in embryonic stem cells.

RESULTS

AIF interacts with CHCHD4. Immunoprecipitation of AIF from human cervical cancer cells, followed by mass spectrometric sequencing of peptides led to the identification of CHCHD4.1, the human equivalent of yeast Mia40 (Tim40) (Chacinska et al., 2004; Naoe et al., 2004) as an AIF-binding partner (Figures 1A, 1B and S1A). Human CHCHD4 is a soluble 16 kDa protein that localizes to the mitochondrial intermembrane space (Hofmann et al., 2005), where it participates to mitochondrial import and catalyzes oxidative protein folding in cooperation with the sulfhydryl oxidase GFER/ALR/Erv1p (Banci et al., 2009; Chacinska et al., 2008; Fischer et al., 2013; Koch and Schmid, 2014). Endogenous AIF and CHCHD4, which both co-localized in mitochondria (Figure S1B), co-immunoprecipitated in conditions in which neither respiratory chain subunits nor GFER were detectable in the complex (Figure 1B and data not shown). Protein pull down experiments revealed direct interaction of recombinant AIF with full-length CHCHD4. This direct protein-protein interaction was not affected by point mutations of cysteine residues in CHCHD4 (Figure 1C) that have previously been implicated in its mitochondrial accumulation and catalytic activity (Banci et al., 2009; Fraga et al., 2014; Hofmann et al., 2005; Koch and Schmid, 2014). A subsequent scan of deletion mutants excluded the participation of the redox-active CX9C-CX9C domain of CHCHD4 in the interaction with AIF (Figures 1D and 1E). Rather, the N-terminal 27 amino acids of CHCHD4 (p1-27N-CHCHD4) were sufficient to interact with AIF (Figures 1D and 1E), and a 27 amino acid synthetic peptide corresponding to this N-terminus competitively disrupted the interaction of recombinant full-length CHCHD4 with AIF (Figure S1C). While the interaction site of CHCHD4 could be precisely mapped to its N-terminus, the interaction site of AIF appeared to be conformational. Accordingly, deletion of different domains of AIF resulted in the generation of a protein that failed to interact with CHCHD4, and only full-length AIF bound to CHCHD4 in cells (Figures S1D and S1E), as well as in GST pull down

experiments (Figure S1F). A pathogenic AIF mutation (E493V) that enhances its apoptotic activity (but does not affect its respiratory activity) (Rinaldi et al., 2012) did not alter the interaction with CHCHD4. In contrast, a mutant AIF that carries a point mutation (G308E) in the binding domain to nicotinamide adenosine dinucleotide (NAD) and causes a severe complex I+IV deficiency (Berger et al., 2011; Ye et al., 2002), exhibited reduced CHCHD4 binding (Figures S1D and 2E). The addition of reduced pyrimidine nucleotides (NADH or NADPH) but not that of their oxidized equivalents (NAD^+ or $NADP^+$) favored the interaction between AIF and full length CHCHD4 (Figure 2A), as well as that of AIF and the N-terminal fragment of CHCHD4, p1-27N-CHCHD4 (Figure 2B). Isothermal titration calorimetry (ITC) confirmed the binding of p1-27N-CHCHD4 to AIF in a 1:1 stoichiometry in the presence of NADH (Figure 2C). NADH failed to affect the structure of p1-27N-CHCHD4 in conditions in which it altered that of AIF (Churbanova and Sevrioukova, 2008), as determined by far-UV circular dichroism (CD) (Figure 2D). NADH did affect the structure of the complex formed by p1-27N-CHCHD4 and AIF, as indicated by subtraction of the CD profiles obtained for the mixture of p1-27N-CHCHD4 plus AIF and the one obtained for AIF, in the absence or presence of NADH (Figure 2D). These results support the notion that the AIF-NADH complex possesses an increased capacity to bind CHCHD4 in comparison with AIF alone and that NADH levels stabilize the interaction between AIF and CHCHD4. Yet another argument in favor of this interpretation could be obtained with the aforementioned pathogenic AIF mutant (G308E), which affects the NADH binding domain of AIF (Figure S1D). In GST pull-down assays involving non-mutated AIF protein immobilized to glutathione-sepharose beads and soluble CHCHD4 protein, the addition of NADH favored the interaction between both recombinant proteins (Figures 2A and 2E). However, the interaction between mutated G308E AIF protein and CHCHD4 was reduced at the basal level and was scarcely enhanced by the addition of NADH (Figure 2E).

Altogether, these results reveal the existence of a novel mitochondrial interactor of AIF, CHCHD4, which directly binds to NADH-AIF complex via its N-terminal domain.

CHCHD4 is required for respiratory chain biogenesis.

Human CHCHD4 and its yeast ortholog MIA40 are involved in the import and assembly of mitochondrial intermembrane space proteins (Banci et al., 2009; Chacinska et al., 2008; Chacinska et al., 2004; Naoe et al., 2004), which then impinge on the assembly and function of the respiratory chain (Allen et al., 2005; Bihlmaier et al., 2007; Chacinska et al., 2004; Dabir et al., 2007). To directly address the possibility that CHCHD4 might affect respiratory chain complexes in mammalian cells, we created mice in which *Chchd4* was inactivated by mutational insertion to express a β-galactosidase-neo (β-GEO) reporter protein under the control of the *Chchd4* promoter (Figure 3A). Heterozygous expression of β-GEO was compatible with normal embryogenesis, revealing ubiquitous expression of the β-GEO transgene throughout development (Figure 3B). Intercrosses of heterozygous mice for homozygous disruption of the *Chchd4* locus yielded no *Chchd4*$^{-/-}$ pups at birth. No *Chchd4*$^{-/-}$ embryos were ever detected after embryonic day (E) E8.5 (Figure 3C). *Chchd4*$^{-/-}$ embryos were always staged E5.5-6 (retarded embryos in Figure 3C), indicating that *Chchd4* deletion causes a developmental arrest coupled to embryonic lethality at the onset of gastrulation. The developmental retardation of *Chchd4*$^{-/-}$ embryos was accompanied by a major defect in the expression of respiratory chain complex I subunit CI-20 (Figures 3D). In accord with this observation, we found that knockdown of CHCHD4 using two distinct, non-overlapping siRNAs led to a similar respiratory chain defect in human osteosarcoma U2OS cells (Figure 4A). Thus, CHCHD4 depletion resulted in reduced protein expression of several respiratory chain subunits, such as CI-20 and CIV-II paralleling functional defects in complexes I and IV

(but not that of complex V) (Figures 4A-4E). As a control, depletion of Mic19, a homologue and potential interactor of CHCHD4 (Darshi et al., 2012; Pfanner et al., 2014), which does not co-immunoprecipate with the AIF/CHCHD4 complex (Figure S2A), failed to mediate similar effects on the stability of the analyzed respiratory chain subunits (Figure S2B).

Taken together, these findings indicate that CHCHD4 plays a central role in the biogenesis of specific respiratory chain complexes.

AIF is required for CHCHD4 protein expression.

The knockdown of CHCHD4 did not diminish the abundance of AIF (Figure 4A). In stark contrast, depletion of AIF led to a reduction of CHCHD4 protein expression (Figure 4A and 4F), yet failed to alter the levels of the related Mic19 protein (Figure S2B). The reduction of CHCHD4 protein in AIF-depleted cells was not accompanied by any change in the levels of CHCHD4-specific mRNA (Figure 4G). The biochemical and functional consequences of AIF depletion phenocopied the biochemical and functional consequences of CHCHD4 depletion, causing selective defects of respiratory chain complexes I and IV (Figures 4A-4E). Moreover, the knockdown of AIF or CHCHD4 had similar effects on the abundance of CHCHD4 substrates (Figures S2C-S2E), NDUFA8 (Szklarczyk et al., 2011), COX17 and DDP1 (Hofmann et al., 2005), which however were not detectable in the AIF/CHCHD4 immunoprecipitate (Figure S2F), presumably because the interaction between CHCHD4 and its substrates is rather transient (Koch and Schmid, 2014; Sideris et al., 2009). Neither AIF nor CHCHD4 affected the abundance of other mitochondrial proteins such as VDAC, PINK and HSP60 (Figures S2C and S2D). The mitochondrial intermembrane space-operating ATP-dependent metalloprotease YME1L (Baker et al.; Jensen and Jasper, 2014) is (one of) the protease(s) that participates to the degradation of CHCHD4 substrates after depletion of AIF.

Indeed, if the siRNA-mediated knockdown of AIF was accompanied by the simultaneous siRNA-mediated knockdown of YME1L, the depletion of CHCHD4 substrates such as COX17 (the copper chaperone for complex CIV) and NDUFA8 (Szklarczyk et al., 2011) (an intermembrane space-localized subunit of complex CI required for the stabilization of the complex) was attenuated (Figure S2E).

AIF depletion by several non-overlapping siRNAs failed to affect the mitochondrial transmembrane potential (Joza et al., 2001), yet caused a reduction of CHCHD4 protein expression with a delay of several days (Figures 5A, 5B and S3C). Re-introduction of AIF expression by means of non-interferable plasmid constructs suppressed the effects of an AIF-specific siRNA on CHCHD4 expression (Figures S3A and S3B). These experiments revealed that both long isoforms of AIF, AIF1 and AIF2 that we characterized in the past (Hangen et al., 2010b), indistinguishably supported the expression of CHCHD4. Mice carrying the hypomorphic *Aif*$^{hq/y}$ mutation (i.e. male mice from the 'Harlequin' strain), which develop signs of neurodegeneration at the adult stage, manifested a progressive CHCHD4 defect in the brain *post partum*. This CHCHD4 defect preceded that of complex I subunits during early adulthood, as detected by quantitative immunoblots (Figures 5C, 5D and S3D). The *Aif*$^{hq/y}$-associated defect in CHCHD4 protein expression occurred solely at the post-transcriptional level (without affecting the abundance of mRNA) and was observed also in other organs from *Aif*$^{hq/y}$ mice (Figures S3E-S3G). AIF-deficient (*Aif*$^{-/y}$) ES cells also manifested reduced CHCHD4 expression as compared to WT controls (Figures 5E and 5F).

Altogether, these findings suggest that AIF acts upstream of CHCHD4 to ensure optimal mitochondrial function at the level of selected protein complexes and that in the absence of AIF, CHCHD4 substrates are unstable and destroyed by proteases such as YME1L.

AIF controls the mitochondrial import of CHCHD4.

As shown above, AIF is required for the expression of CHCHD4 protein. AIF depletion has no effect on the half-life of CHCHD4 after translation blockade with cycloheximide (CHX), excluding that the absence of AIF enhances the degradation of CHCHD4 (Figure S4A). Therefore, we investigated the potential effect of AIF on CHCHD4 import using a novel assay system. U2OS cells were transfected with a plasmid coding for the *Escherichia coli* biotin ligase BirA (de Boer, 2003) targeted to the mitochondrial intermembrane space by means of a mitochondrial localization sequence (MLS) derived from the first 120 N-terminal residues of AIF (Otera et al., 2005). This plasmid was dubbed MLS-BirA. Simultaneously, cells were co-transfected with a CHCHD4 fusion construct (CHCHD4-b) that carried in its C-terminus a specific 15-amino acid acceptor peptide flanking a lysine residue that constitutes an effective acceptor for site-specific biotinylation by BirA (Figure 6A). MLS-BirA was exclusively detected in mitochondria (Figure 6C) facing the intermembrane space (data not shown) and effectively biotinylated CHCHD4-b in a translation-dependent (CHX-repressible) manner (Figure 6B). Knockdown of AIF reduced the biotinylation of CHCHD4-b as it reduced the overall abundance of CHCHD4-b (Figure 6D). Thus, knockdown of AIF negatively affected the expression of endogenous CHCHD4, the overall protein levels of CHCHD4-b, as well as those of biotinylated CHCHD4-b, to a similar level (Figure S4B), in line with the interpretation that the translation-dependent biotinylation of CHCHD4-b by MLS-BirA reflects its overall abundance. Kinetic experiments during which biotin was removed from culture media before CHX treatment and the translation was restarted by CHX removal in the presence of biotin, confirmed that AIF depletion (by means of two distinct siRNAs) reduced the rate of CHCHD4-b biotinylation (Figures 6E and S4C).

Next, we explored the putative implication of AIF in translation-coupled import by measuring co-translation within a fusion construct composed by N-terminal CHCHD4 and C-terminal

unstable GFP (Li et al., 1998), that were separated by the 2A self cleaving peptide (de Felipe et al., 2006) (Figure 6F)**.** The 2A self-cleaving peptide provokes ribosomal "skipping", causing an apparent cleavage at this site without the need of proteolysis (de Felipe et al., 2006). We transfected this new construct into cells and observed that the co-translation of both recombinant CHCHD4 (upstream of 2A) and GFP (downstream of 2A) was attenuated when AIF was depleted. This effect has been quantified in two ways, namely by assessing the GFP-dependent fluorescence of cells (by flow cytometry) and by measuring the expression of GFP by immunoblot, yielding concordant results (Figure 6F-I). Hence, by interfering with the co-translational import of CHCHD4 into mitochondria, AIF depletion also reduced the translation of GFP in this system. In contrast, AIF depletion failed to affect the translation of another, co-transfected control plasmid (MLS-BirA) that also codes for a mitochondrial protein, underscoring the specificity of the effects. Altogether, this new assay corroborates the hypothesis that AIF controls the translation-coupled mitochondrial import of CHCHD4.

We conclude that AIF is required for translation-coupled mitochondrial import of CHCHD4.

Enforced mitochondrial CHCHD4 import restores respiration in AIF-deficient cells. The aforementioned results are compatible with a molecular pathway in which AIF facilitates CHCHD4 import through direct protein-protein interactions, and CHCHD4 acts downstream of AIF to assure optimal abundance and function of respiratory chain supercomplexes. To directly explore this possibility, we attempted to restore the expression of CHCHD4 in AIF-depleted cells, by modifying its mitochondrial import pathway and enforcing its attachment to the inner membrane with an appropriate mitochondrial localization sequence (MLS) derived from the first 120 N-terminal residues of AIF(Otera et al., 2005), fused to its N-terminus (Figure 7A). The expression level of this MLS-CHCHD4 chimera (MC) was not affected by

one of the AIF-specific siRNA (AIFc), yet was strongly reduced by another siRNA (AIFa) that targets the stretch of the mRNA sequence corresponding to the MLS shared by AIF and MC (Figures 7B and S5A). Importantly, MC was able to revert the defects in DDP1 and respiratory chain complex subunits (CI-20, GRIM 19 from complex I; CIV-II from complex IV and the CIV-chaperone COX17) secondary to AIF depletion (Figures 7B and S5A). In contrast, a control construct (MB, composed of the same MLS from AIF and an irrelevant protein, BirA) was unable to reverse the respiratory chain subunit defect induced by AIF depletion (Figure 7B). Mutation of all functional cysteines contained in CHCHD4 abrogated the capacity of the MC construct to restore the mitochondrial defects of AIF-deficient cells (Figure S5B), underscoring the relevance of the cysteine-dependent oxidoreductase activity of CHCHD4.

Stable transfection with MC (but not with MB) could also restore the expression of CI-20 in undifferentiated *Aif* $^{-/y}$ ES cells, as well as in *Aif* $^{-/y}$ embryoid bodies (EB) that are formed from ES cells upon depletion of leukemia-inhibitor factor (LIF), a cytokine that usually maintains the pluripotency and represses the differentiation of ES cells (Murray and Edgar, 2001). This effect was obtained both in normoxic (Figures 7C and 7D) and hypoxic (Figures S5C and S5D) conditions. Concomitantly, MC (but not MB) improved the function of respiratory chain complexes I and IV in *Aif* $^{-/y}$ ES cells (Figure 7E) and restored the capacity of the cells to form cavitating embryoid bodies (EBs) upon LIF removal. MC-expressing *Aif* $^{-/y}$ EBs underwent cavitation in conditions in which MB-expressing or untransfected (not shown) *Aif* $^{-/y}$ EBs failed to undergo cavitation (Figure 7F and 7G). In sharp contrast, MC had no impact on the formation and size of EBs (Figure 7G).

Altogether, these results indicate that restoring CHCHD4 levels can reverse the phenotype of *Aif* $^{-/y}$ ES cells with respect to their metabolic and cell death phenotypes.

Discussion

AIF has two distinct functions, one that coined its name (as a pro-death protein) and another that is linked to its physiological location in mitochondria. The deletion or depletion of AIF reduces cell death rates in response to endogenous stimuli (development, aging) or external stressors. Moreover, AIF deficiency provokes respiratory chain defects in all species investigated in this respect, from yeast to man (see Introduction). Indeed, in humans, mutations in AIF manifest as familial X-linked diseases that have either of two different phenotypes. First, AIF mutations can cause a severe pediatric mitochondriopathy linked to reduced expression and function of respiratory chain complexes (Berger et al., 2011; Ghezzi et al., 2010). Second, the so-called Cowchock syndrome is caused by a mutation of AIF that increases its cytocidal potential (Rinaldi et al., 2012). In the present work, we reveal a series of experimental results that explain how the absence of AIF can provoke a respiratory defect. Our data plead in favor of a molecular pathway in which deficient expression of AIF in mitochondria entails reduced import of another phylogenetically conserved protein, CHCHD4, which in turn is required for mediating optimal mitochondrial import of a series of respiratory chain proteins. Conversely, restoration of CHCHD4 levels reverses metabolic and phenotypic alterations driven by AIF deficiency.

There are several lines of evidence that place CHCHD4 in the pathway linking AIF deficiency to the deficient biogenesis of respiratory chain complexes. First, there is chronological evidence in favor of this hypothesis. In Harlequin mice, which bear a retroviral insertion in the first intron of AIF, causing a reduction of its expression, the defect in CHCHD4 becomes evident before a major reduction of respiratory chain complex I is detectable at the post-natal stage. Second, there is functional evidence in favor of a precise mechanistic hierarchy between AIF and CHCHD4. Thus, several different approaches to reduce or eliminate AIF expression, namely, siRNA-mediated depletion, hypomorphic mutation or deletion by

homologous recombination, all cause a reduction in the abundance of CHCHD4. Conversely, knockdown of CHCHD4 fails to affect the expression of AIF in several distinct cell lines. Hence, AIF is required for CHCHD4 expression, not *vice versa*. Third, the phenotypes of cells exhibiting reduced or null expression of AIF and CHCHD4 are similar. Depletion of both proteins causes defective expression of several mitochondrial proteins, including components of the respiratory chain complexes I and IV and DDP1. In contrast, a vast array of other proteins specifically expressed in this organelle is not affected by AIF or CHCHD4 depletion. This applies to respiratory chain complex V, as well as to outer membrane-associated proteins such as VDAC, PINK or the matrix-localized HSP60. Moreover, genetic deletion of AIF and CHCHD4 caused early embryonic lethality, supporting the idea that both proteins play essential (and probably similar) roles in bioenergetics. Thus, AIF (Brown et al., 2006) and CHCHD4 knockout embryos manifest a defect in complex I. Fourth, when the import of CHCHD4 into the mitochondrial intermembrane space is rendered independent from AIF, namely by fusing the MLS of AIF with CHCHD4, the resulting chimeric protein dubbed "MC" (for mitochondrion-targeted CHCHD4), is able to overcome the respiratory chain defect that is normally detectable in AIF-depleted or AIF-deleted cells. This experimental observation closes the cycle of argumentation. CHCHD4 depletion is indispensable for the phenotypic manifestation of an AIF-related defect in oxidative phosphorylation.

How does AIF affect the expression levels of CHCHD4? Having excluded the possibility that AIF might influence the levels of mRNA coding for CHCHD4, we evaluated the hypothesis that AIF would affect the import of CHCHD4 into mitochondria. To test this possibility, we determined the capacity of AIF to regulate the import of CHCHD4 protein in human cells. For this, we first created a new experimental system in which a bacterial biotinylase specifically targeted to the intermembrane space was combined with a version of CHCHD4 (CHCHD4-b)

that carries a biotinylation motif, meaning that the degree of CHCHD4-b *in vivo* biotinylation reflects its localization to mitochondria. Kinetic experiments led to the conclusion that the import of CHCHD4 into mitochondria was translation-dependent (because it only occurred in the absence of cycloheximide) and that it was affected by the abundance of AIF. In AIF-deficient cells the biotinylation (and hence import) of CHCHD4-b by the intermembrane space-targeted biotinylase was reduced. Second, we developed a protein co-translation assay in which unstable (non-mitochondrial) GFP was placed downstream of CHCHD4-b on the same mRNA, in the same open reading frame, finding that AIF deficiency reduced the abundance of GFP in conditions in which it affected CHCHD4-b expression. Translation-dependent import is not a rarity (Weis et al., 2013). However, to the best of our knowledge this is the first report that AIF can affect such an import reaction.

How might the presence of AIF in mitochondria facilitate the translation-dependent import of CHCHD4? It appears plausible that AIF affects the import of CHCHD4 through a specific effect. Indeed, AIF does not affect the import/abundance of VDAC in mammalian cells. AIF was found to physically interact within cells with CHCHD4, as determined by co-immunoprecipitation assays of endogenous AIF and CHCHD4 and confirmed by GST pulldown experiments, proving that the AIF/CHCHD4 interaction is a direct one, not requiring additional proteins. No specific interaction domain within AIF could be identified by truncation and deletion scanning, suggesting that this interaction relies on the entire tertiary structure of the AIF protein. In line with this interpretation, we found that NADH (or NADPH but not NAD^+ nor $NADP^+$) enhanced the binding of AIF to CHCHD4, knowing that only the holo-AIF protein (but none of the truncation or deletion mutants studied here) does possess NADH or NADPH reductase activity (Mate et al., 2002; Ye et al., 2002). Moreover, a mitochondriopathy-associated point mutation occurring within the NADH-binding domain of AIF (Berger et al., 2011), reduced the interaction with CHCHD4, and the addition of NADH

barely ameliorated the formation of the complex. In contrast to AIF, the binding of CHCHD4 involved a relatively short domain. The extreme N-terminal, 27-amino acid-long fragment of CHCHD4 was necessary and sufficient for it to interact with AIF, and mutational analyses revealed that the redox function of CHCHD4 was irrelevant to the CHCHD4/AIF interaction. The N-terminus of CHCHD4 is unstructured when examined by NMR spectroscopy (Banci et al., 2009). However, far-UV circular dichroism (CD) data and isothermal titration calorimetry (ITC) profiles were compatible with the idea that this peptide firmly interacts with AIF, in particular in the presence of NADH. Future co-crystallization of these interactors may confirm the hypothesis that the N-terminus of CHCHD4 acquires a defined structure as it binds to AIF. Irrespective of these incognita, it appears plausible that AIF may 'drag' the CHCHD4 N-terminus into the mitochondrial intermembrane space, thereby facilitating its import. Once the entire CHCHD4 protein has been taken up into this compartment, it may adopt its mature conformation and catalyze oxidative protein folding by virtue of its capacity to form mixed disulfides with protein substrates (Milenkovic et al., 2009; Sideris et al., 2009), thereby contributing to optimal mitochondrial biogenesis (Yang et al., 2012). It is possible, but still remains to be determined, that CHCHD4 bound to AIF maintains its catalytic activity by transiently interacting with its partner enzyme (Erv1) and its substrates. Obviously, it will be interesting to investigate the possibility that CHCHD4 mutations could provoke similar phenotypes as those described for its interactors, AIF and Erv1 (Di Fonzo et al., 2009).

Unexpectedly, we found that the phenotype of AIF-null (*AIF*$^{-/y}$) ES cells with regard to cavitation (Feraud et al., 2007; Joza et al., 2001) was secondary to the mitochondrial dysfunction. Following up the finding that partial inhibition of the respiratory chain complex I with rotenone was sufficient to abolish cavitation in WT EB (Feraud et al., 2007), we observed that transfection of *AIF*$^{-/y}$ ES cells with mitochondrion-targeted CHCHD4, a maneuver that corrected the mitochondrial defect, also restored the capacity of *AIF*$^{-/y}$ ES cells

to undergo cavitation upon in vitro differentiation. At present, the precise molecular mechanisms that link oxidative phosphorylation to cavitation-associated programmed cell death remain elusive.

Altogether, we conclude that the effect of AIF on the biogenesis of the mitochondrial respiratory chain complexes is mediated by its physical and functional interaction with CHCHD4, an essential mitochondrial intermembrane protein.

Experimental procedures.

Statistical analyses: GraphPad Prism 5.0d Software was used for statistical analyses. Groups were compared by one-way ANOVA followed by Bonferroni post analyses or unpaired two-tailed t-test with Welch's corrections. The criterion for statistical significance was set at $p < 0.05$. Data are expressed as mean ± SEM.

Extended experimental procedures are included in the supplemental information section.

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Figure Legends

Figure 1. Physical interaction between AIF and CHCHD4.

(A) Identification of CHCHD4/MIA40 as an AIF-binding protein by immunoprecipitation of AIF (or as a control an isotype-matched purified rabbit IgG). Mass spectrometry-identified peptides that match CHCHD4 are underlined.

(B) Co-immunoprecipitation of endogenous AIF and CHCHD4, as performed on human cancer cell lysates. The presence of the indicated protein in the immune complex was checked by immunoblot. Whole cell extract (Input) represents 25% of the input.

(C-E) Interaction of recombinant CHCHD4 with AIF. (C) Interaction of GST-tagged cysteine point mutants of CHCHD4 with recombinant AIF (rAIF). (D and E) Interaction of GST-tagged full length and truncation mutants of CHCHD4 either with AIF contained in the lysate of U2OS cells (D) or with rAIF (E). The indicated CHCHD4 derivatives (schematic presentations in bottom of each panel) were immobilized on beads and then evaluated for their capacity to bind AIF. Experiments were performed at least three times, yielding similar results.

Figure 2. Impact of NADH on the stabilization of AIF/ CHCHD4 complex. (A) Interaction of GST-tagged AIF with recombinant CHCHD4 (rCHCHD4) in the presence of pyrimidine nucleotides. GST-tagged AIF (103-613) was immobilized on beads, pre-incubated (+) or not (-) with pyrimidine nucleotides (NAD, NADP, NADH and NADPH) and then evaluated for its capacity to bind rCHCHD4. Experiments were performed at least three times, yielding similar results.

(B) Interaction of GST-tagged mutants of CHCHD4 with AIF/NADH. The indicated WT or mutant GST-tagged CHCHD4 recombinant proteins (schematic presentation in the right panel) were immobilized on beads and evaluated for their capacity to bind recombinant AIF

(103-613) that was pre-complexed (+) or not (-) with NADH. All experiments were performed at least three times, yielding similar results.

(C) Isothermal titration calorimetry (ITC) profiles of AIF interaction with the synthetic peptide p1-27N-CHCHD4, in presence of NADH. Left panel (top) displays the raw data for sequential injections of a p1-27N-CHCHD4 solution into PBS (supplemented with NADH) used as blank and hAIF(Δ1-103) solution (supplemented with NADH). The bottom left panel shows the plot of the heat evolved (kilocalories) per mole of p1-27N-CHCHD4 added to the buffer solution (o) and into the hAIF(Δ1-103) solution (●). Right panel (top and bottom) display the titration of hAIF(Δ1-103) with p1-27N-CHCHD4, corrected for the heat of p1-27N-CHCHD4 dilution, against the molar ratio of p1-27N-CHCHD4 to hAIF(Δ1-103). The data were fitted to a single-site binding model. All plots are representative of at least three independent determinations**.**

(D) Secondary structure analysis of p1-27N-CHCHD4 / hAIF(Δ1-103) interaction using far-UV Circular Dichroism (CD) in absence (●; Co.) or presence of NADH (o; NADH).

No protein panel, CD spectra collected on the buffer alone. p1-27N-CHCHD4 panel, CD spectra collected on p1-27N-CHCHD4 solution. Addition of the flavonoid does not induce any structural change on the peptide. AIF panel, CD spectra collected hAIF(Δ1-103) solution. As expected, the presence of NADH largely influences the structure of the protein. AIF+ p1-27N-CHCHD4 panel, CD spectra recorded on combined p1-27N-CHCHD4 and hAIF(Δ1-103) solution. AIF+ p1-27N-CHCHD4/After background subtraction panel, the same spectra shown in AIF+ p1-27N-CHCHD4 panel after subtraction of the hAIF(Δ1-103) contribution presented in AIF panel. As shown, the peptide undergoes a large conformational rearrangement due to the interaction with the protein but the conformational change is larger in the presence of the cofactor. All spectra are representative of at least three independent determinations.

(E) Pull down of GST-tagged WT AIF or mutated AIF^{G308E} (Berger et al., 2011) with the recombinant His-tagged CHCHD4 protein, in presence or absence of NADH. GST-tagged AIF (103-613) WT or mutated G308E (schematic localization of G308E mutation in the NADH binding domain of AIF is presented in the top panel) was immobilized on beads, pre-incubated (+) or not (-) with NADH and then evaluated for their capacity to bind rCHCHD4. The Immunoblot (middle panel) was realized using the indicated antibody. The membrane was stained with a reversible blue protein stain for GST-AIF loading control (bottom panel). Experiments were performed at least three times, yielding similar results.

Figure 3. Impact of CHCHD4 knockout on respiratory chain complexes during embryogenesis. (A) Schematic representation of the gene trap strategy used for the insertional mutagenesis of *CHCHD4* gene. The insertion of the βGEO cassette, at the vicinity of exon 2, disrupts the endogenous coding sequence and allows the expression of a β-galactosidase-neo reporter mRNA that is initiated in the first exon of *CHCHD4* and reports the transcriptional activity of the locus.

(B) X-Gal staining of mutant embryos from E5.5 to E9 revealed a widespread expression of CHCHD4, with the exception of the visceral endoderm at the earliest stages. EPC: ectoplacental cone; YS: yolk sac. Scale bars: 200 μM.

(C) CHCHD4 invalidation leads to embryonic lethality at gastrulation. Embryos were genotyped by PCR amplification and staged by somite counting or according to Downs and Davis (Downs and Davies, 1993) criteria at pre-somitic stages (top and bottom tables). Embryos indicated as "retarded embryos" were staged E5.5-6. In addition to homozygous ($CHCHD4^{-/-}$) embryos that were nearly always staged E5.5-6, some heterozygous embryos ($CHCHD4^{+/-}$) staged at E5.5-6 were also recovered from the litters containing homozygous knockout embryos (bottom table). Scale bar: 200 μM.

(D) Impact of *CHCHD4* mutation on the expression of respiratory chain complexes protein subunits. Extracts of individual embryos, staged E5.5-6, were analyzed by immunoblot (left panel). The abundance of indicated proteins in the mutant embryo (-/-; deficient for the expression of CHCHD4) was quantified by image analysis and normalized compared to the levels of same proteins in the embryo proficient for the expression of CHCHD4 (+/+) (right panel). Actin was used as a loading control. nd: not detectable.

Figure 4. Impact of AIF and CHCHD4 depletion on the levels of mitochondrial proteins.

(A to G) Extracts of U2OS cells (triplicates), subjected to the transfection with siRNAs that deplete AIF (AIFa), CHCHD4 (CHCHD4a, CHCHD4b) or Emerin as a control (Co.1), were analyzed by immunoblot for the abundance of the indicated proteins (A), and the relative expression levels of proteins (with respect to complex V subunit CV-α) were quantified by image analysis (B, C, F). In (F) the ratio of AIF or CHCHD4 protein to CV-α is set to 100%. in control siRNA-transfected cells. The relative deficiency of complex I and IV activity (with respect to complex V) was analyzed by spectrophotometric measurement of individual respiratory chain complexes activities (D, E). Relative mRNA levels encoding AIF or CHCHD4 were determined by quantitative RT-PCR, defining the ratio of AIF or CHCHD4 to three housekeeping mRNAs in control siRNA-transfected cells set to 100% (G). Values are means ± SEM of triplicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ calculated by Ordinary One Way ANOVA followed by Bonferroni's post-analyses.

Figure 5. Kinetic ordering of the AIF/CHCHD4 pathway.

(A and B) Extracts of U2OS cells, transfected with the indicated siRNAs for 1 to 4 days, were analyzed by immunoblot for the expression of AIF and CHCHD4 (A) and the relative abundance of each protein was quantified by image analysis followed by normalization compared to the level of Actin (B). Results were expressed as a percentage of control value

(siRNA Co.4) defined as 100 %. Values are means ± standard deviation of duplicates. * p<0.05, ** p<0.01, *** p<0.001 calculated by ANOVA followed by Bonferroni's post-analyses.

(C and D) The kinetic impact of the HQ mutation on AIF, CHCHD4 and complex-I-20 protein expression was determined in brain samples collected at the indicated day *post partum*. Triplicate samples from wild type ($Aif^{+/y}$) and mutant ($Aif^{hq/y}$) mice were analyzed by immunoblot (C) and the relative abundance of each protein was determined by quantitative image analysis followed by normalization compared to Actin protein levels. Results were expressed as a percentage of control value, with P60-WT defined as 100 % (D). Values are means ± SEM of triplicates. * p<0.05, ** p<0.01, *** p<0.001 calculated by ANOVA followed by Bonferroni's post-analyses.

(E and F) Mouse embryonic stem cell (mES) lines AIF WT ($Aif^{+/Y}$; 2 independent cell lines) and AIF deficient ($Aif^{-/Y}$; 2 independent cell lines) were analyzed by immunoblot for the abundance of the indicated proteins (E) and the relative expression levels of proteins were quantified by image analysis and normalized compared to the level of GAPDH (F).

Figure 6. Regulation of CHCHD4 translation/import by AIF.

(A) Schematic representations of CHCHD4-b, the biotinable recombinant CHCHD4 that carries in its C-terminus a 15-amino acid acceptor peptide (GLNDIFEAQ**K**IEWHE) flanking a lysine residue that constitutes an effective acceptor for site-specific biotinylation by BirA and MLS-BirA, the mitochondrion-targeted BirA, which is addressed to the organelle thanks to a fusion with the mitochondrial localization sequence (MLS) derived from AIF1 (residues 1-120).

(B) Assessment of translation-coupled import of CHCHD4 in the mitochondrion. U2OS cells, stably overexpressing CHCHD4-b and MLS-BirA proteins, were plated and incubated in the absence (DMSO) or presence of cycloheximide (CHX) for 16h (schematic protocol in the

figure). Then, cells (3) and (4) were washed and re-incubated, only for 1h, in absence (3) or presence (4) of CHX. Finally, the biotinylation of CHCHD4-b and the abundance of the indicated proteins were analyzed on triplicate samples and compared to that of control samples incubated for 17h in absence (1; DMSO) or presence (2; CHX) of CHX.

(C) Mitochondrial localization of MLS-BirA. The subcellular localization of MLS-BirA was assessed and compared to that of a HA-tagged BirA by immunoblot analysis of the indicated proteins in the mitochondrial and cytosolic extracts.

(D) Impact of AIF knockdown on the translation-coupled import of CHCHD4 in the mitochondrion. U2OS cells stably overexpressing CHCHD4-b and MLS-BirA were transfected with control siRNAs (Co.2 and Co.3) or with an AIF-specific siRNA (AIFb) and submitted to the CHX treatment protocol described in (D). Only extracts prepared from cells incubated in presence of CHX for 16h and then washed and re-incubated for 1h in absence of CHX (16h CHX + 1h fresh medium) are presented in the figure. The biotinylation of CHCHD4-b and the abundance of the indicated proteins, in the lysates of AIF knockdown cells are analyzed on triplicate samples and compared to that of triplicate samples prepared with cells transfected with control siRNA Co.2 and Co.3.

(E) Impact of AIF knockdown on the kinetics of the translation-coupled import of CHCHD4 into mitochondria. U2OS cells stably overexpressing the biotinable CHCHD4-b and MLS-BirA were transfected with control siRNAs (Co.1) or an AIF-specific siRNA (AIFc) and submitted to a successive biotin-deprivation and cycloheximide treatment protocol presented in a schematic format in the figure. 16h after cycloheximide treatment, cells were washed and incubated for the indicated times in presence of fresh CHX-free medium supplemented with 8μM biotin. Finally, extracts were analyzed for the biotinylation of CHCHD4 and the levels of the indicated proteins.

(F-I) Impact of AIF knockdown on the translation-coupled expression of CHCHD4-b and GFP. (F) The construct CHCHD4-b-2A-GFP (Schematic representation) is designed to

produce a single fusion ORF composed by an N-terminal CHCHD4 and a C-terminal unstable GFP protein (Li et al., 1998), which are linked among each other by a 2A self cleaving peptide (de Felipe et al., 2006). (G) During the translation phase, the self-processing of the 2A peptide allows the production of CHCHD4-b that is imported in the mitochondrion and bitotinylated by MLS-BirA, while the GFP protein is diffused everywhere in the cell. Biotinylated CHCHD4-b (Bio) is revealed by indirect immunofluorescence, in presence of phycoerythrin-coupled streptavidin. Scale bar, 10µm. (H-I) U2OS cells stably co-transfected with CHCHD4-b-2A-GFP and MLS-BirA plasmids were transfected with control siRNA (Co.3) or with AIF-specific siRNAs (AIFa, AIFb, AIFc) or with CHCHD4-specific siRNA (CHCHD4a). Four days post-transfection, the abundance of GFP in AIF knockdown cells is analyzed by fluorescence (flow cytometry) (H) or by immunoblot, in parallel to the indicated proteins (I) and compared to that of samples prepared with cells transfected with control Co.3 siRNA or CHCHD4 siRNA that knocks-down directly the fusion RNA CHCHD4-b-2A-GFP. The graph 6H shows the percentage of GFP positive cells observed after transfection with the indicated siRNA. Values are means ± SEM of five independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ calculated by ANOVA followed by Bonferroni's post-analyses.

Figure 7. Phenotypic reversal of the AIF defect by mitochondrion-targeted CHCHD4.

(A) Schematic representation of mitochondrion-targeted CHCHD4 (MC) and BirA (MB), which both share an identical mitochondrial localization sequence (MLS) derived from AIF1 (residues 1-120).

(B) Impact of MC and MB on the abundance of mitochondrial proteins of AIF-depleted cells. U2OS cells were stably transfected with either MC or MB and then subjected to the knockdown of Emerin (control, Co.1) or AIF, using either a siRNA that only targets AIF (AIFc) or another siRNA (AIFa) that targets both AIF and MC or MB (which all contain

identical, AIF-derived N-termini). The abundance of the indicated proteins was determined by immunoblot.

(C and D) Impact of MC and MB on the abundance of mitochondrial proteins in AIF null mutant cells. Mutant (*Aif*$^{-/y}$) murine cells were stably transfected with MC or MB and grown in the conditions of high oxygen tension (normoxia). Then, lysates were prepared from ES cells (grown in the presence of LIF) or embryoid bodies (EB4 and EB10; generated respectively by withdrawal of LIF for 4 and 10 days) and analyzed for the expression of the transgene and respiratory chain related proteins. The relative expression level of CI-20 subunit protein was quantified by image analysis and normalized compared to the level of CV-α protein subunit (D). Values are means ± SEM of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ calculated by One Way ANOVA followed by Bonferroni's post-analyses. Data are representative of at least three experiments that yielded comparable results.

(E) Histograms showing the respiratory function of mutant (*Aif*$^{-/y}$) ES cells stably transfected with MC or MB. Values are means ± SEM of triplicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ calculated by t-test (with Welch's correction).

(F and G) Phenotype of embryoid bodies derived from wild type (*Aif*$^{+/y}$) and mutant (*Aif*$^{-/y}$) ES cells stably transfected with MC or MB. Representative pictures are shown for embryoid bodies of the indicated genotype (bar = 200 μm) (F). Histograms showing the percentage of cystic EBs (G top panel) and the measure of their perimeter are presented (G bottom panel).

Interaction between AIF and CHCHD4 regulates respiratory chain biogenesis

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Supplemental Information.

Legends to Supplemental Figures

Supplementary figure 1. Physical interaction between AIF and CHCHD4.

(A) Immunoprecipitation of AIF (or as a control an isotype-matched purified rabbit IgG), revealed by SDS/PAGE electrophoresis.

(B) Mitochondrial localization of human CHCHD4. The localization of CHCHD4 was assessed by subcellular fractionation and immunoblot analysis of the mitochondrial (M), cytosolic (C) and nuclear (N) and total (Total) extracts.

(C) Immunoprecipitation of AIF (or as a control an isotype-matched purified rabbit IgG) from U2OS cells incubated or not with CHX for 16hr. Immunoblots were realized using the indicated antibodies.

(D) Competitive disruption of the interaction between GST-AIF (retained on glutathione sepharose beads) and recombinant His-tagged CHCHD4 protein in the presence of a synthetic peptide corresponding to the first 27 N-terminal residues of CHCHD4 (peptide p1-27), or a mutated peptide (peptide p1-27 delta) that lacks the first 6 N-terminal residues. All experiments have been performed at least three times, yielding similar results.

(E) Co-immunoprecipitation of Flag-tagged WT AIF or mutated AIF^{E493V} (Rinaldi et al., 2012) or AIF^{G308E} (Berger et al., 2011) with the recombinant HA-tagged CHCHD4 protein, as performed on human cancer cell lysates overexpressing the recombinant CHCHD4 and AIF proteins. Immunoblots were realized using the indicated antibodies.

(F) Co-immunoprecipitation of endogenous CHCHD4 (2) or as a control an isotype matched IgG, (3) with a panel of Flag-tagged full length or C-terminal truncation mutants of AIF (schematic presentation in the right panel). The red star indicates the full length Flag-tagged AIF that was co-precipitated with the endogenous CHCHD4.

(G) Interaction of GST-tagged truncation mutants of AIF with CHCHD4 contained in the lysate of U2OS cells. The indicated AIF deletion mutants (schematic presentation in the right panel) were immobilized on beads and were then evaluated for their capacity to retain CHCHD4 protein.

Supplementary figure 2. Cofractionation of AIF and CHCHD4 from bacterial extracts.

(A-G) Representative gel filtration elution profiles at 280 nm of: purified AIF(Δ1-103) (A), GST- CHCHD4/MIA40 (B), AIF(Δ1-103) and GST- CHCHD4/MIA40 mixture (C), GST (E), and GST and AIF(Δ1-103) mixture (F), were loaded onto a Superdex™ 200 10/300 GL column as described above, and eluted at a flow rate of 0.5 mL/min. (D): Overlay of chromatograms A, B and C. (G), Representative SDS-PAGE of the main peak fractions (a, b, c, e, f(I) and f(II)) on a 15% SDS-PAGE gel, under reducing conditions, are visualized by Coomassie staining. Experiments were repeated three times yielding similar results.

Supplementary figure 3. Impact of AIF, CHCHD4 and YME1L depletion on the levels of mitochondrial proteins.

(A) Co-immunoprecipitation of endogenous AIF and HA-tagged recombinant CHCHD4 protein (anti-HA IP), as performed on human cancer cell lysates. The presence of indicated proteins in the immune complex was verified by immunoblot.

(B-D) U2OS cells were transfected with specific siRNAs that deplete AIF (AIFa and AIFb), Mic19, CHCHD4 (CHCHD4a, CHCHD4b, CHCHD4c) or Emerin as a control (Co.1). The abundance of the indicated proteins was analyzed on duplicate (B) or single samples (C) by immunoblot, and the relative expression levels of proteins were quantified by image analysis

and normalized compared to the level of Actin protein (D). Results were expressed as percentage of control values, control being the levels of proteins in cells transfected with Emerin (Co.1). Values are means ± SEM obtained for the indicated gene-specific siRNA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ calculated by One Way ANOVA followed by Bonferroni's post-analyses. Data are representative of at least three experiments that yielded comparable results.

(E) U2OS cells were transfected with siRNAs that deplete either AIF (AIFb) or YME1L, or both siRNA combined (AIFb/YME1L). The abundance of the indicated proteins was measured on duplicate samples by immunoblot and compared to that of control siRNA (Co.2).

(F) Co-immunoprecipitation of endogenous AIF and CHCHD4 protein (or as a control an isotype-matched purified IgG), as performed on human cancer cell lysates. The presence of CHCHD4 substrats COX17 and DDP1 was checked in the immune complex by immunoblotting.

Supplementary figure 4. Kinetic ordering of the AIF/CHCHD4 pathway.

(A and B) To exclude off-target effects, U2OS cells were simultaneously transfected either with an AIF-specific siRNA (AIFb), which targets the 3'untranslated region [UTR] of the AIF mRNA (A) or with an AIF-specific siRNA (AIFa) that depletes exclusively the endogenous and the flag-tagged AIF1 (Hangen et al., 2010) (B) and plasmids encoding either of the two AIF isoforms (Flag-tagged AIF1 or AIF2, lacking the 3'UTR (Hangen et al., 2010)) or vector only (A and B). The abundance of the indicated proteins was analyzed on duplicate samples by immunoblot.

(C) U2OS cells were transfected either with an AIF-specific siRNA (AIFb) or with a control siRNA Co.1 and four days post-transfection, whole cell extracts (WCE), mitochondrial (M)

and cytoplasmic (C) fractions were prepared and the abundance of the indicated proteins was analyzed by immunoblot.

(D) Kinetic impact of the HQ mutation on AIF, CHCHD4 and complex-I-20 protein expression. Brain samples were collected at the indicated day *post partum*. Duplicate samples from wild type (*Aif* $^{+/y}$) and mutant (*Aif* $^{hq/y}$) mice were analyzed by immunoblot for the abundance of the indicated proteins.

(E-G) The impact of the HQ mutation on AIF, CHCHD4 and respiratory chain complexes subunit protein expression was determined on various organs (brain, spleen and liver) obtained from adult mice. Triplicate samples from wild type (*Aif* $^{+/y}$) and mutant (*Aif* $^{hq/y}$) mice were analyzed by immunoblot (E) and the relative abundance of each protein was determined by quantitative image analysis and normalized compared to the level of Actin protein (F). Results were expressed as a percentage of control value, control being the abundance of each protein in the WT organ. In addition, relative mRNA levels of *Aif* or *Chchd4* were determined by quantitative RT-PCR, defining the ratio of *Aif* or *Chchd4* to three housekeeping mRNAs in control organs (G). For F and G values are means ± SEM obtained for triplicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ calculated by One Way ANOVA followed by Bonferroni's post-analyses.

Supplementary figure 5. Regulation of CHCHD4 translation/import by AIF.

(A) Analysis of CHCHD4 degradation profile. U2OS cells stably overexpressing a recombinant biotinable CHCHD4 (CHCHD4-b) were transfected either with control siRNA Co.2 or an AIF-specific siRNA AIFb and incubated in presence of the protein translation inhibitor cycloheximide (CHX) for 0 to 5h. Then, the abundance of the indicated proteins in the whole cell extracts was analyzed on triplicate samples by immunoblot.

(B) Impact of AIF knockdown on the translation-coupled import of CHCHD4 in the mitochondrion. U2OS cells stably overexpressing the biotinable CHCHD4-b and the

mitochondrion-targeted BirA (MLS-BirA) were transfected with control siRNA (Co.2) or an AIF-specific siRNA (AIFb). Four days later cells were re-plated and submitted to the CHX treatment protocol schematically presented in the figure. Finally, for all the extracts, the biotinylation of CHCHD4-b and the abundance of the indicated proteins were analyzed on triplicate samples.

(C) Impact of AIF knockdown on the kinetics of translation-coupled import of CHCHD4 in the mitochondrion. U2OS cells stably overexpressing the biotinable CHCHD4-b and the mitochondrion-targeted BirA (MLS-BirA) were transfected with control siRNA (Co.2) or AIF-specific siRNAs (AIFc: left panel or AIFb: right panel) and submitted to a successive biotin-deprivation and cycloheximide treatment protocol schematically presented in the figure. 16h after cycloheximide treatment, cells were washed and incubated for the indicated times in presence of fresh medium supplemented with 8µM biotin. Finally, extracts prepared from the samples were analyzed for the biotinylation of CHCHD4 and the levels of the indicated proteins.

Supplementary figure 6. Phenotypic reversion of the AIF defect by mitochondrion-targeted CHCHD4.

(A) Impact of MC on the abundance of mitochondrial proteins in AIF-depleted cells. U2OS cells were stably transfected with either MC or the empty vector (mock) and then subjected to the knockdown with a control siRNA (Co.2) or with AIF-specific siRNAs that target only AIF (AIFc) or both AIF and MC (AIFa)(Note that AIF and MC contain identical, AIF-derived N-termini coding for the mitochondrial localization sequence). The abundance of the indicated proteins was determined by immunoblot.

(B) Impact of cysteine mutated-MC on the abundance of mitochondrial proteins in AIF-depleted cells. U2OS cells were stably transfected with a variant of MC (MCcysmut) that is mutated for all functional cysteines contained in CHCHD4 (C4S, C53S, C55S, C64S, C74S,

C87S and C97S). Cells were then subjected to the knockdown of Emerin (control, Co.1) or AIF, using either a siRNA that only targets AIF (AIFc) or another siRNA (AIFa) that targets both AIF and MC (which all contain identical, AIF-derived N-termini coding for the mitochondrial localization sequence). The abundance of the indicated proteins was determined by immunoblot.

(C and D) Impact of MC and MB on the abundance of mitochondrial proteins in AIF null cells. Mutant (*Aif* $^{-/y}$) cells were stably transfected with MC or MB and grown in the conditions of low oxygen tension (hypoxia). Then, lysates were prepared from ES cells (grown in the presence of LIF) or embryoid bodies (EB4 and EB10; generated respectively by withdrawal of LIF for 4 and 10 days) and analyzed for the expression of transgenic and respiratory chain related proteins. The relative expression level of CI-20 subunit protein was quantified by image analysis and normalized to the level of CV-α (D). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ calculated by One Way ANOVA followed by Bonferroni's post-analyses. Data are representative of at least three experiments that yielded comparable results.

EXTENDED EXPERIMENTAL PROCEDURES

Antibodies: Antibodies against the following proteins were used: actin (mouse mAb; CHEMICON); AIF (mouse mAb; Santa Cruz and rabbit pAB; Cell Signaling and Santa Cruz); CHCHD4 (rabbit pAB; Santa Cruz; mouse mAB; Santa cruz and rabbit pAB; Sigma); CI-SU20 (NDUFB8) (mouse mAb; Mitosciences); NDUFA8 (rabbit mAb, abcam); VDAC (rabbit pAb; Cell Signaling); Hsp60 (mouse mAb; Stressgen); GAPDH (rabbit pAB; Santa cruz); P53 (mouse mAB; Santa cruz); caspase 3 (rabbit pAB; Cell Signaling); PARP (rabbit pAB; Cell Signaling); Mic19 (goat pAB; abcam); BirA (chicken pAB; abcam); total human OXPHOS proteins (mouse mAB cocktail; Mitosciences); total rodent OXPHOS proteins (mouse mAB cocktail; Mitosciences); ATP5A (mouse mAB; abcam); GRIM19 (mouse mAB; abcam); COX17 (rabbit pAB; abcam); DDP1 (rabbit pAB; PTG); Flag tag (mouse mAb; SIGMA); HA tag (rabbit mAb; Cell Signaling); YME1L (rabbit pAB; PTG); TURBO GFP (rabbit pAB; Evrogen).

Plasmids and siRNA: Recombinant plasmids pCMV-AIF1-3xFlag and pCMV-AIF2-3xFlag were constructed as described (Hangen et al., 2010). All FLAG-tagged c-terminal deletion mutants of human AIF1 were cloned in the vector pCMV-3x FLAG-CMV-14 (Sigma) using EcoRI and KpnI sites. Indicated AIF deletion mutants were subcloned in the bacterial expression vector pGEX-6P using EcoRI and NotI sites. Substitutions E493V (Rinaldi et al., 2012) and G308E (Berger et al., 2011) were introduced in the pCMV-AIF1 or pGEX-AIF expression plasmid using a site-directed mutagenesis kit (QuickChange Site-directed Mutagenesis kit, Stratagene), and respectively the primers GATGTTGGCTATGtAGCTATTGGTCT and TACGATTATCGGTGaGGGCTTCCTTGG. For the construction of the recombinant prokaryotic plasmid pETM11-AIF (103-613), a fragment of human AIF cDNA, corresponding to the residues 103 to 613 (hAIF 103 - 613),

was PCR-amplified using the primers Bbs1-5' (GAAGACTCCATGGGGCTGACACCAGAACAG) and EcoR1-3' (GAATTCTCAGTCTTCATGAATGTT) and finally cloned into Nco1 and EcoR1 sites of the vector pETM-11 (EMBL).

Recombinant plasmids for the expression of human CHCHD4 in mammalian cells (pCMV-CHCHD4-HA and pCMV-CHCHD4-bio) or in bacteria (pT7-His-CHCHD4 and pT7-GST-CHCHD4) were purchased from Genocopoeia. Indicated CHCHD4 deletion mutants were subcloned in the bacterial expression vector pGEX-6P using EcoRI and NotI sites. Cysteine residues present in CHCHD4 were mutated into serines by using a site-directed mutagenesis kit (QuickChange Site-directed Mutagenesis kit, Stratagene) and primers as described (Hofmann et al., 2005).

The bacterial biotin ligase expression vector 3XHA-BiRA pBUDneo is a kind gift of Dr John Strouboulis (de Boer, 2003). For the construction of the plasmid pBUDneo-MLS-BirA (pBUD-MB) the segment coding for the 3XHA tag was removed from 3XHA-BiRA pBUDneo using NotI and BglII restriction enzymes (New England Biolabs) and replaced by the PCR-amplified N-terminal segment of human AIF1 that codes for the first 120 amino acids (Hangen et al., 2010; Otera et al., 2005). The first N-terminal 40 residues of AIF1 are necessary for the mitochondrial targeting, while the segment corresponding to residues 66-84 are required for the sorting to the IMS (Otera et al., 2005). Moreover, it has been clearly established that when fused to other proteins, the first N-terminal 120 residues of AIF allow the fusion protein to be correctly localized in the IMS, in a comparable manner to full length AIF protein (Otera et al., 2005). For the construction of pBUDneo-MLS-CHCHD4 (pBUD-MC), the sequence coding for BirA was removed from pBUDneo-MLS-BirA (pBUD-MB) using BglII and XhoI restriction enzymes (New England biolabs) and replaced by the PCR

amplified cDNA corresponding to the open reading frame of the human CHCHD4.

Coding sequences for MLS-CHCHD4 (MC) and MLS-BirA (MB) were PCR-amplified from their respective pBUDneo host vectors using Platinum Taq Polymerase (Invitrogen) and the following primers: 5'-CGTAACTCCCGGGATCCTAAATGTTCC-GGTGTGGAGG-3' (left) and 5'-CGTAACTGCGGCCGCTTAGGGATAGGCTTACCTTCG-3' (right). PCR products were agarose gel-purified using the Geneclean system (MP Biomedicals), and digested with XmaI and NotI restriction enzymes (New England Biolabs). Purified digested products were then introduced in an Epstein-Barr Virus-based episomal vector (kind gift from D. Biard, CEA, France) at AgeI-NotI sites, downstream of the strong and constitutive CAG promoter. The resulting pEBV-MC and pEBV-MB plasmids were sequence-verified to confirm the absence of any mutation in the corresponding coding sequences.

For the construction of the recombinant plasmid CHCHD4-b-2A-GFP, the DNA sequence including the fusion ORF CHCHD4-b-2A-GFP was chemically synthesized and cloned in the restriction sites Nhe1 and BamH1 of pCDNA_3.1 vector (GeneArt; Life Technologies). In this recombinant plasmid, the 2A cleavage sequence of porcine teschovirus-1 that encodes a 22 residues peptide (Kim et al., 2011) is flanked at N-terminus by CHCHD4-b (see above) and at C-terminus by GFP-des. For the purpose of the study, GFP was rendered less stable by fusing to its c-terminus the degradation domain of mouse ornithine decarboxylase (residues 422-461) (Li et al., 1998); des denotes "destabilized". In this system, the apparent cleavage that allows the production of two discrete products (CHCHD4-b and GFP-des), is due to a ribosome skipping and a consequent inhibition of covalent linking between residues glycine (amino acid 21) and the c-terminal proline (amino acid 22) in the 2A peptide (NPG_{21}/P_{22}) (de Felipe et al., 2006). In this context, as a result of the cleavage, the C-terminal protein (GFP-des) will start with a proline residue.

For RNA interference experiments the following siRNA sequences were used: for negative control: Co.1: CCG UGC UCC UGG GGC UGG G [dT][dT]; Co.2: AUG CAG AAC UCC AAG CAC G [dT][dT]; Co.3: GCG AUA AGU CGU GUC UUA C [dT][dT] (Delettre et al., 2006); Co.4: GCC UAG UAU ACU UCU UAA U [dT][dT]. For human AIF: AIFa: GGG CAA AAU CGA UAA UUC U[dT][dT]; AIFb: GCA GAC UUU CUC UGU GUA U[dT][dT]; AIFc: GCA UGC UUC UAC GAU AUA A[dT][dT](Delettre et al., 2006); For human CHCHD4: CHCHD4a: GCA UGG AUU GAU ACU GCC ATT [dT][dT]; CHCHD4b: GGA AGG AUC GAA UCA UAU U[dT][dT]; CHCHD4c: CGA GGA GCA UGG AUU GAU ATT [dT][dT] (Applied Biosystems). For human Mic19: GAG UCA CCA CUG AAC AAU A[dT][dT] (Applied biosystems). For human YME1L: GAUGCAUUUAAAACUGGUUUU[dT][dT] (Griparic et al., 2007).

RNA Isolation and gene expression analysis by quantitative real-time PCR (qRT-PCR): Total RNA from wild type (wt) or harlequin (Hq) mutant mice organs were extracted using Precellys homogenizer (Bertin) and RNA isolation kit from Qiagen. RNA from human cells in culture were extracted using PARIS kit (Ambion). All RNA samples were then stored at -80°C.

The quantification of RNA samples was achieved using the Nanodrop ND-1000 Spectrophotometer and the integrity of the RNA was verified using the Agilent 2100 Bioanalyzer with the Eukaryote Total RNA Nano assay. One microgram of total RNA was reverse-transcribed in a 20 µl final reaction volume using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems) following the manufacturer's instructions. For the human and mouse AIF1, the following primers and Taqman MGB probes were custom-made by Applied Biosystems: for human AIF1 : forward (hAIF1F): 5'-GGCAAAATCGATAATTCTGTGTTAGTC-3'; reverse (hAIFR): 5'-

CCACCAATTAGCAGGAAAGGAA-3'; probe (hAIFcp): 5'-TGTTTCTGTTCTGGTGTCAG-3'; for mouse Aif1 : forward (mAif1-F): 5'-CGAGCCCGTGGTATTCGA-3'; reverse (mAif1R): 5'-CCATTGCTGGAACAAGTTGC-3'; probe (mAifSc): 5'-ACGGTGCGTGGAAG-3'. TaqMan® probes were labeled with 6-FAM at the 5' end and with a nonfluorescent MGB quencher at the 3' end. Each probe was combined with different forward and reverse primers (see list) for AIF1 quantification. TaqMan® gene expression assays for human CHCHD4 (Hs 01027804-g1), mouse CHCHD4 (Mm01183497-g1), 18S ribosomal RNA (Hs99999901-s1), Gapdh (Mm99999915-g1), Pgk1 (Mm00435617-m1), Tbp (Mm00446973-m1) were from Applied Biosystems. Quantitative PCR reactions were performed using ABI Prism 7900 HT sequence detection system (Applied Biosystems). Real-time q-PCR amplifications were carried out (10 min 95°C followed by 45 cycles of 15 sec 95°C and 1 min 60°C). Technical replicates were performed for each biological sample. For Aif1 and Chchd4 expression quantification, reference genes were selected using the TaqMan® Mouse Endogenous Control Arrays. Briefly, 16 housekeeping genes were tested in triplicate for each sample. The most stable genes were selected by analyzing results with GeNorm and Normfinder functions in Genex 4.3.8 (MultiD, Göteborg, Sweden). The geometric mean of 3 housekeeping genes (Gapdh, Pgk1 and Tbp) was used to normalize gene expression levels of Aif1 and Chchd4 for further analysis of q-PCR experiments with the relative quantification method.

Peptide synthesis and characterization. Reagents for peptide synthesis including Fmoc-protected amino acids and resins, activation and deprotection reagents, were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland) and InBios (Naples, Italy). Solvents for peptide synthesis and HPLC analyses were purchased from Romil (Dublin, Ireland); C18 Biobasic columns for peptide analysis and the LC-MS system were from ThermoFisher (Milan, Italy). Solid phase peptide synthesis were performed on a fully automated

multichannel peptide synthesizer Syro I (Multisyntech, Germany) following the Fmoc methodology (Fields and Noble, 1990). Preparative RP-HPLC purification was carried out on a Waters Quaternary Gradient Module, equipped with a Waters UV/Vis detector and with a X Bridge^{TM} Prep BEH 130 column (19x50 mm; 185 μm). MS analyses were carried out on a Bruker HCT ETD II ultra PTM Discovery System equipped with an ESI source maintained at 5 kV and 300 °C. Narrow bore 50x2 mm C18 BioBasic LC-MS columns were used for these analyses.

Isothermal titration calorimetry (ITC). ITC experiments were conducted at room temperature (298 K) on a MicroCal ITC200 system. Protein samples were dialyzed against PBS pH 7.4 with or without 100 μM NADH and thoroughly degassed before each experiment. The concentration of hAIF(Δ1-103) in the sample cell was 0.018 mM; a solution of p1-27N-CHCHD4 at 0.371 mM was injected into the cell at 2 minute intervals using a 100 μL syringe. Nineteen 2.0 μL injections were carried out to complete the titration. The first injection consisted of 0.4 μL of p1-27N-CHCHD4. The resulting data were processed using the software provided by the manufacturer (Origin 7, MicroCal Software, Inc.).

Circular dichroism (CD) spectroscopy. CD spectra were collected using a JASCO J-810 spectropolarimeter (Tokyo, Japan). The CD parameters were: wavelength scan range, 190–260 nm; data pitch, 0.2 nm; continuous scan mode, 10 scans of each sample; scan speed, 100 nm/min; 1 sec response; band width, 2 nm. All spectra were recorded after an accumulation of three runs. Spectra were processed using the means movement smoothing parameter within the Spectra Manager software. CD spectra were recorded in 10 mM phosphate buffer, pH 7.4 and in the same buffer supplemented with NADH 100 μM. The p1-27N-CHCHD4 peptide was tested at 50 μM, whereas the protein AIF(Δ1-103) at 2.5 μM.

Assessment of respiratory chain activities: Respiratory chain activities were measured on subconfluent mES (overexpressing MB or MC) or U2OS cells (4 days after knocking-down AIF or CHCHD4 with the selected siRNA) permeabilized with 0.01% digitonin. Rotenone-sensitive NADH quinone reductase (complex I; EC 1.6.5.3), oligomycin-sensitive ATP hydrolase (complex V; EC 3.6.3.14), cyanide-sensitive cytochrome *c* oxidase (complex IV; EC 1.9.3.1), malonate-sensitive succinate cytochrome c reductase (an indirect measure of succinate dehydrogenase – EC 1.3.5.1 - limiting for this activity; EC 1.3.99.1), glycerol cytochrome c reductase (an indirect measure of glycerol 3 phosphate dehydrogenase, - EC 1.1.5.3 - limiting for this activity); antimycin-sensitive quinol cytochrome *c* reductase (complex III; EC 1.10.2.2), were spectrophotometrically measured using a pseudo dual-wavelength Varian CARY50 spectrophotometer (Victoria, Australia) as described previously (Benit et al., 2006). Results are expressed as means ± SEM of activity ratios as these have been established to be the most sensitive markers to detect partial defects in the respiratory chain. All measurements were performed at 37°C. Protein levels were determined by the method of Bradford with bovine serum albumin as a standard. All chemicals were analytical reagent grade from Sigma Chemical Company.

Cell culture and transfection: Human Osteosarcoma U2OS cells (ATCC n°HTB-96) as well as cervix carcinoma Hela cells (ATCC n°CCL-2) were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies) and 1% penicillin/streptomycin. Plasmid and siRNA transfections were performed using Lipofectamine-2000 reagent (Life Technologies) by following manufacturer's procedure. Pools of stably transfected U2OS cells were established by cotransfection with any combination of the plasmids described above and the pPuro plasmid (Clontech) followed by selection in the presence of 0.5µg/ml of puromycin (Invivogen) and 0.5mg/ml of geneticin.

Mouse embryonic stem cell culture and transfection: Mouse embryonic stem cell (mES) lines AIF wt (*Aif* $^{+/Y}$) and AIF deficient (*Aif* $^{-/Y}$) were propagated on 0.1% gelatin (Sigma) coated dishes in mES medium based on High Glucose/Glutamax-I DMEM supplemented with 15% fetal calf serum (Hyclone), 1mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids, 100 µM β-mercaptoethanol (all from Life technologies), and 1000 U/ml leukemia inhibitory factor (ESGro, Millipore). For transfection, 2 x 10^4 mES cells per well of a 12-well plate were seeded. The next day, plasmid DNA (pEBV-MB or pEBV-MC) was mixed with FuGENE® HD (Promega) at a 2:1 ratio according to the manufacturer's instructions and 50 µl of complex were added per well. The next day medium was changed and selection with 0.5 µg/ml puromycin (Invitrogen) started 48 hours later. Four stably transfected cell lines expressing respectively MB (MB-*Aif* $^{+/Y}$ and MB-*Aif* $^{-/Y}$) and MC (MC-*Aif* $^{+/Y}$ and MC-*Aif* $^{-/Y}$) were established and then routinely propagated in mES medium supplemented with 0.5 µg/ml puromycin. Medium was changed daily and the cells were grown at 37°C in atmospheric conditions with additional 5% CO_2.

Embryoid body (EB) formation: For the formation of EBs, 2 x 10^5 mES cells per well were seeded in 6-well Ultra Low Attachment plates (Corning) in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 15% fetal calf serum (Hyclone), 2mM L-glutamin (Invitrogen), 100 U/ml penicillin, 100µg/ml streptomycin, 50 µg/ml ascorbic acid (Sigma), 450 µM monothioglycerol (Sigma) 200 µg/ml iron-saturated transferrin (Sigma) and 0.3 µg/ml puromycin. Medium was changed every 4 days and plates were incubated at 37°C in atmospheric conditions supplemented with 5% CO_2. Proportion of cystic EBs was evaluated by morphology.

Protein half-life assessment: U2OS cells stably overexpressing the recombinant biotinable

CHCHD4 (CHCHD4-b) protein and the mitochondrion-targeted BirA (MLS-BirA) were transfected either with control or AIF-specific siRNAs. Four days later, transfected cells were replated in 24 wells plates (300.000 cells/well) and incubated for 16h before being treated with the protein translation inhibitor cycloheximide (CHX) (Sigma Aldrich) (30 µg/ml) for 0 to 5h. Triplicates, for each time point, were each extracted with 200 µl of 2xSB (4% SDS, 20% glycerol, 125 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol) and boiled for 3 min. Finally, 10µl of each sample were resolved with PAGE/SDS (NUPAGE/SDS; life technologies) electrophoresis and analyzed by immunoblot.

Translation-coupled mitochondrial import of CHCHD4: U2OS cells stably overexpressing the biotinable CHCHD4 (CHCHD4-b) and the mitochondrion-targeted BirA (MLS-BirA), transfected or not with siRNA, were plated in 24 wells plates (300.000 cells/well) and incubated 24h later, in the presence of the reversible protein translation inhibitor CHX for 16h, in order to eliminate the old CHCHD4-b polypeptides. Then, for the purpose of reactivating protein translation, CHX was washed away using PBS and cells were re-incubated, only for 1h, in a fresh medium free of CHX. Finally, triplicates for experimental or control samples, were each extracted with 200 µl of 2xSB (4% SDS, 20% glycerol, 125 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol), boiled for 3 min, and 10µl of each sample were resolved with PAGE/SDS (NUPAGE/SDS; life technologies) electrophoresis and analyzed by immunoblot.

The kinetic of in vivo biotinylation of CHCHD4-b was assessed using the above-mentioned protocol except that, 24h before the addition of CHX, the regular medium was replaced by a biotin-depleted one. After 16h of CHX treatment (in the absence of biotin), cells were washed with PBS and incubated for 0 to 60 min with the biotin-depleted CHX-free medium supplemented with 8µM Biotin (Sigma Aldrich). Biotin-depleted medium was prepared by

supplementing the DMEM medium with a heat-inactivated biotin-free FBS. In brief, 10 ml of FBS were rotated at 4°C in presence of Monomeric Avidin-conjugated Agarose beads (1.5 ml of a 50% slurry; Thermo scientific). After 24h rotation, beads were settled by gravity and the supernatant that corresponds to biotin-depleted FBS was transferred to fresh tubes and used for the preparation of the above-mentioned medium.

To assess the in vivo biotinylation of CHCHD4-b, after transferring the SDS-PAGE-separated proteins, the nitrocellulose membrane (Bio-Rad) was first blocked by incubation with 1% BSA in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1h and then incubated for a further 1h with a horseradish peroxidase-conjugated streptavidin (Sigma Aldrich). Finally the biotin-streptavidin binding was detected with the ECL chemiluminescence detection kit (GE Healthcare).

GST pull down: GST fusion proteins were produced and recovered on glutathione-Sepharose 4B beads (GE Healthcare) according to Kaelin et al. (Kaelin et al 1991). When indicated, after glutathione affinity purification, the GST moiety of the fusion protein was cleaved off after digestion with the PreScission protease (GE Healthcare) and the pure recombinant AIF (103-613) was recovered in the post-digestion supernatant. For competition experiments, GST-AIF protein was first recovered on glutathione-Sepharose beads, then preincubated with synthetic peptides CHCHD4 1-27 (MSYCRQEGKDRIIFVTKEDHETPSSAE) or CHCHD4 1-27 delta (EGKDRIIFVTKEDHETPSSAE), before being incubated with His-CHCHD4 recombinant protein.

Cell fractionation: U2OS cells overexpressing HA-tagged BirA or MLS-BirA were processed for mitochondrial and cytosolic fraction preparation according to Hangen et al. (Hangen et al., 2010). For the analysis of human CHCHD4 sub-cellular by immunoblot, cell

fractions were prepared either using a cell fractionation kit (Mitoscience) or a protocol based on differential centrifugation. Briefly, after siRNA transfection, U2OS cells were harvested in the isolation buffer (IB) (2mM Hepes-KOH, pH 7.4; 70 mM sucrose, 220mM mannitol and protease and phosphatase inhibitors (ROCHE)), homogenized using a Teflon pestle and centrifuged at 1300 g at 4°C to discard debris and unbroken cells. Mitochondria were pelleted by centrifugation at 12000 g at 4°C for 30 min. The supernatant was further centrifuged for 60 min at 65000 g at 4°C to obtain the cytosolic fraction. Finally, after adding 1% SDS, proteins in all samples were quantified and subjected to immunoblot analyses.

Tissue extract preparation for immunoblot. Wild type (wt) or Harlequin (Hq) mice were anesthetized and killed by decapitation at the indicated age. All the dissected organs were snap-frozen and then homogenized, using Precellys homogenizer (Bertin), in an ice-cold RIPA 1X buffer (Sigma Aldrich), supplemented with protease (EDTA- free protease inhibitor tablet - Roche Applied Science) and phosphatase inhibitors (PhosSTOP phosphatase inhibitor tablet - Roche Applied Science).

Immunoblotting: U2OS cells (transfected or not) were lysed at 4°C for 30 min, in NetN-120 buffer (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1mM EDTA, 0.5% Igepal) supplemented with protease inhibitors and phosphatase inhibitors. The lysate was centrifuged for 10 min at 13,000 *g* to eliminate cell debris. The proteins present in the supernatant were quantified (Bio-Rad DC protein assay) and subjected to direct immunoblot analyses or immunoprecipitation, GST pull down followed by immunoblot analysis.

Immunoprecipitation: In brief, protein G-Sepharose CL-4B beads (50 µl of a 50% slurry, GE Healthcare) were coated with the indicated antibodies (1 to 2 µg per immunoprecipitation), by incubating for 2 to 3h at 4°C in 800 µl of NetN-120 buffer (20mM

Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% Igepal). The antibody-coated beads were washed in NetN-120 buffer and incubated for 2h at 4°C with the cell lysate, in a final volume of 800 µl. Beads were washed three times with NetN-120 buffer supplemented with protease and phosphatase inhibitors and after a last wash with NetN-120, samples were finally resolved directly by SDS-PAGE (NUPAGE; Invitrogen) after boiling in 1xSB (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol). After electrophoresis, the gel was subjected either to immunoblot analysis or to silver staining procedure (Silver stain plus kit and procedure- Biorad) to visualize co-precipitated protein bands.

Identification of AIF associated proteins by mass spectrometry: Specific protein bands co-purifying with the anti-AIF antibody were excised from the gels, subjected to in-gel tryptic digestion, concentrated with ZipTip µC18 pipette tips (Millipore) and co-eluted directly onto a MALDI target. MALDI-MS and MALDI-MS/MS were performed on an Applied Biosystems 4700 MALDI TOF/TOF. The interpretation of both the MS and MS/MS data was carried out with the GPS Explorer software (Version 3.6, Applied Biosystems). A combined MS peptide fingerprint and MS/MS peptide-sequencing search was performed against the NCBI database without taxon restriction using the MASCOT search algorithm. These searches specified trypsin as the digestion enzyme, carbamidomethylation of cysteine as fixed modification, partial oxidation of methionine and phosphorylation of serine, threonine, and tyrosine as variable modifications, and allowed for one missed trypsin cleavage. The monoisotopic precursor ion tolerance was set to 30 ppm and the MS/MS ion tolerance to 0.3 Da. MS/MS peptide spectra with a minimum ion score confidence interval $\geq$ 95% were accepted; this was equivalent to a median ion score cut off of approximately 35 in the data set. Protein identifications were accepted with a statistically significant MASCOT protein search score $\geq$ 75 that corresponded to an error probability $p < 0.05$ in our data set.

Animals: All procedures and animal experimentation protocols (project authorization n°2012-076) were reviewed and deemed acceptable by Registered Ethical Committee n°26 and carried out in the animal facility of Gustave Roussy (Official agreement number E-94-076-11).

Generation of *Chchd4* heterozygous mice: Mutant *Chchd4* animals were constructed by Texas Institute for Genomic Medicine (TIGM) using a gene-trapping strategy. The gene trap vector (Omnibank Vector 76) contained a lacZ-neomycin resistance fusion cassette (β-GEO). A mouse (C57/BL6 background) was cloned from an ES cell line (IST11943B12) that contained the retroviral insertion in the *Chchd4* gene (chromosomal position chr 6: 91 433 279 – 91 433 280). In brief, the mutant ES cells were microinjected into C57BL/6 host blastocysts to generate germ line chimeras.

Mouse genotyping: 3 to 4 weeks after weaning, tail snip DNA was extracted using the Maxwell16 mouse tail DNA purification kit (Maxwell). Using the AmpliTaq Gold master Mix (Applied Biosystems #4316753), PCR was performed for the detection of the wt or mutant *Chchd4* allele interrupted by a gene trap vector. The wt allele (213 bp PCR product) was amplified using the Primers IST11943B12-F (TGGGCTGGTTAGTCAGTGATTGG) and IST11943B12-R (GTGCTCCTCATAGGGATCATTGG) and the mutant allele (205 bp PCR product) was amplified using IST11943B12-R and LTR2 (AAATGGCGTTACTTAAGCTAGCTTGC).

Embryo manipulation: To obtain embryos, heterozygous males and females were crossed and the morning vaginal plug observation was considered as embryonic day (E) 0.5. Pregnant females were sacrificed by cervical dislocation. Embryos from E5.5 to E7.5-8 were staged

according to (Downs and Davies, 1993). Embryos between E8 and E10 were staged by somite counting. Dissected embryos were washed with PBS in order to remove all the contaminating maternal tissues. After staging and photographing, individual embryos were genotyped using the DNA extraction and PCR amplification protocols mentioned above or analyzed by immunoblot.

β-Galactosidase staining: After PBS wash, dissected embryos were fixed, for 30 minutes at room temperature, in PBS supplemented with 0.2% paraformaldehyde, 0.1M PIPES pH6.9, 2mM $MgCl_2$, 5mM EGTA. Then, after washes in PBS, embryos were incubated, overnight at 37°C, with an X-Gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside, 5 mM Potassium Ferricyanide, 5 mM potassium Ferrocyanide, 2mM $MgCl_2$, 0.02% Nonidet P-40, 0.01% Na Deoxycholate, in PBS).

Embryo image acquisition: Freshly dissected and wholemount X-Gal stained embryos were observed on Olympus SZX12 stereomicroscope and images were acquired with the DP50 Olympus digital camera (Analysis© software). Images were processed with the Adobe Photoshop CS software.

Automated Cell image acquisition and analysis: Five x 10^3 U20S cells transfected or not with CHCHD4-b-2A-GFP plasmid were seeded per well in 96-well black imaging plates (Greiner Bio-One, Monroe, CA, USA). After 24hr incubation at 37°C, cells were fixed with 4% PFA for 20 min., permeabilized with 0.2% Triton for 5 min and blocked with 5% BSA for 1hr. Finally, fixed cells were incubated for 1hr with PE-conjugated streptavidin (eBioscience) and 2 μM Hoechst 33342. Images were acquired on ImageXpress Micro XL automated microscope (Molecular Devices, Sunnyvale, CA, USA) equipped with a Spectra X light engine (Lumencor, Beaverton, OR, USA), multiple excitation and emission filters (Semrock, Rochester, NY, USA) and a scientific CMOS digital camera. Four view fields were taken per

well at 20 X magnification, followed by image processing with MetaXpress V5.0 (Molecular Devices). According to utilized biosensor, nuclei were segmented, and a second cytoplasmic Region of Interest (ROI) was defined. This ROI was used for the identification of GFP positive and streptavidin-PE-stained cells that overexpressed biotinylated CHCHD4-b (streptavidin binds biotin with high affinity) in mitochondria.

Flow cytometry: Data were acquired on a BD-LSRII (BD Biosciences) and FACSDiva software (BD Biosciences) was used for analyses.

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